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Comparison of Conventional Cytogenetic and Fluorescence In Situ Hybridization Results of Prenatal Aneuploidy Screening

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Background and Aim: Prenatal diagnosis of chromosome abnormalities is usually performed on amniotic fluid samples. Aneuploidy screening by fluorescence in situ hybridization (FISH), although not an alternative for conventional cytogenetics, has become a useful tool.

Materials and Methods: We compared the results of FISH and conventional cytogenetic analysis applied on 51 amniotic fluid samples between January 2002 and December 2005, in our Cytogenetics Laboratory. Amniocytes were extracted using 5 cc of the sample, and direct FISH was applied using an AneuVysion Assay Kit (Vysis). The remaining fluid samples were used for chromosome analysis by conventional methods after long-term cell cultures.

Results: The results of both methods were compatible for 48 patients. For the 2 patients, although FISH results were normal, structural abnormalities were detected by the conventional cytogenetic method. In one patient, we detected mosaic trisomy 21 by FISH, whereas a 47,XX,+21 karyotype was identified in all cells that were examined by the conventional cytogenetic method.

Conclusions: During genetic counseling, it is important to inform patients who undergo aneuploidy screening by FISH about the advantages and disadvantages of the method to help them acknowledge the results in the event that an abnormality is identified in the karyotype.

Key Words: Aneuploidy, prenatal diagnostic tests, FISH, conventional cytogenetics

Anöploidi Taramasında Konvansiyonel Sitogenetik ve Floresan İn Situ Hibridizasyon Bulgularının Karşılaştırılması

Giriş ve Amaç: Amniyon sıvısından kromozom eldesi ve analizi, doğum öncesinde kromozomal hastalıkların tanısında en sık kullanılan yöntemdir. Floresan in Situ Hibridizasyon (FISH) yöntemi ise konvansiyonel sitogenetik için bir alternatif olmamakla birlikte yararlı bir tanı aracıdır.

Yöntem ve Gereç: Bu çalışmada 2002-2005 yılları arasında sitogenetik laboratuarımıza anöploidi taraması için gönderilen 51 olgunun amniyon sıvısı örnekleri üzerinde uygulanan FISH ve konvansiyonel sitogenetik analiz sonuçları karşılaştırmalı olarak değerlendirildi. Gönderilen materyalden her olguda 5 cc lik sıvı ayrılarak amniyositler elde edildi ve bunlara Aneuvysion Assay Kit (Vysis) probu kullanılarak direkt FISH yöntemi uygulandı. Kalan sıvı uzun dönem hücre kültürü sonrasında konvansiyonel yöntemlerle kromozom eldesi için kullanıldı.

Bulgular: Sonuçlar karşılaştırıldığında, 48 hastada FISH ve konvansiyonel sitogenetik yöntem sonuçları uyumlu bulundu. 2 hastada ise FISH ile anöploidi taraması sonucu normal olarak değerlendirilmesine karşın konvansiyonel sitogenetik yöntemle yapısal anomali saptandı. Direk FISH yöntemi ile mozaik trizomi 21 saptanan bir hastada ise konvansiyonel sitogenetik yöntemle incelenen tüm hücrelerde 47,XX,+21 karyotipi saptandı.

Sonuç: Sonuç olarak, FISH yöntemi kullanılarak anöploidi taraması yapılan hastaların genetik danışma ile yöntemin avantaj ve dezavantajları açısından bilgilendirilmesi, karyotipte anormallik saptanması durumunda, bulguları kabullenmeleri açısından önem taşımaktadır.

Anahtar Sözcükler: Anöploidi, prenatal tanı testleri, FISH, konvansiyonel sitogenetik

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Introduction

In every pregnancy, there is a risk of having an aneuploid fetus. Twenty years ago, the extra risk beyond the general risks was attributed to advanced maternal age (AMA). Today, the values achieved from maternal serum screenings and ultrasonographic examinations of the fetus are also used effectively for the identification of increased risks. The parameters utilized, on the one hand, augment the false positivity in risk assessment, whereas, on the other hand, they help determine other risky situations the fetus might be in. In pregnancies in which aneuploidy risk is identified, a common approach is to obtain chromosomes from the amniotic fluid and to analyze them. By this method, which can be applied during the 16^{th}-20^{th} week of pregnancy, a diagnosis can be reached by identifying the chromosomal set-up of the fetus in 14-21 days. This period, which is necessary for the diagnosis, can be very stressful for families who are worried about the real risk, especially when the method is applied in the later weeks of pregnancy and in cases of high risk accompanied by abnormalities in fetal USG. In applications of post-amniocentesis (AS) culture, approximately 2% of the cultures are unsuccessful due to cells that cannot adapt themselves to the environment (1). In such a case, the family is informed, and either the AS procedure is repeated or cordosynthesis (CS) is applied. In overcoming such difficulties, aneuploidy screening with FISH, the result of which can be obtained in 3 days at most and which does not require chromosome extraction, is crucial. In this method, chromosomes 13, 18, 21, X and Y, which account for 80% of all aneuploidies, are evaluated quantitatively in the nucleus. However, this research does not allow the conducting of structural evaluation or assessments for other chromosomal defects.

In the current study, we compared the conventional and the molecular cytogenetic results of 51 cases referred to our laboratory because of an increased aneuploidy risk.

Materials and Methods

Patients: We included 51 cases that had been sent to our laboratory between January 1, 2002, and December 31, 2005, for aneuploidy screening after amniocentesis by conventional and molecular cytogenetic methods. The indications were increased risk for Down Syndrome (DS) (15) or trisomy 18 (T18) (1) in maternal serum triple screening test (TST), increased T18 (2) risk by dual screening test (DST), abnormalities in fetal USG (18), advanced maternal age (AMA) (9), increased risk of DS due to AMA + TST (4), and DS presence in the previous child (2).

Conventional Cytogenetics: Five milliliters of each amniotic fluid sample sent to our laboratory was kept in sterile conditions for direct FISH. The remaining sample was divided into 2; the cell suspension was added to Amniomed (Biochrom, Germany) medium in 2 separate flasks and they were left in a humid environment at 37 ºC with 5% CO_2 for long-term culture. On the first day, the cultures were examined for viability and infection. On the seventh day, the medium in the flasks was taken along with the non-attached cells and added to a new flask, and new medium was added to the first set of flasks. As the colonization and mitotic activities of the cells became adequate, chromosome harvesting by conventional cytogenetic methods was performed, and the number and structure of the chromosomes were analyzed by conducting GTG and C banding, where necessary (2). The karyotype was interpreted according to the International System for Human Cytogenetic Nomenclature (ISCN, 2005) by analyzing at least 5 metaphases structurally and 20 metaphases numerically for each individual (3).

Molecular Cytogenetics: The cells were obtained from the 5 ml aliquot of the amniotic fluid after centrifugation. They were prepared for direct FISH by treatment with 1X Trypsin/EDTA, 75 mM KCl and Carnoy's fixative, respectively.

The fixed cells were spread on 2 separate microscope slides. After being dried at room temperature, they were kept in 1% pepsin solution for 20 min at 37 °C. Then they were washed with distilled water and PBS solution, kept in 2X SSC for 5 min, and washed with 70%, 85%, and 90% ethyl alcohol, consecutively. The hybridization was performed under the recommended conditions with LSI 13/21 (Vysis, Aneuvysion, 32-161075) and CEP 18, X, Y (Vysis, Aneuvysion, 32-161075) probe mixtures. After hybridization, the slides were washed with the recommended solutions, and DAPI-II was applied.

For analysis, a minimum of 50 and a maximum of 100 nuclei were evaluated from each slide using the appropriate probe filters. Cells that did not provide clear signals were not taken into consideration. Identification of 2% abnormal cells was considered false positive, whereas identification of an abnormality rate of 15% or above was considered true abnormality.

Results

Findings regarding 51 cases that had been referred to our laboratory for aneuploidy screening are given in Table. One of our cases was a twin pregnancy (case numbers 19 and 20), while the others were single fetuses. Five cases out of the 51 were diagnosed as aneuploidy (4 cases) and euploidy (1 case) by FISH. In all of these cases, aneuploidies were also determined by the conventional cytogenetic method. Among these 5 abnormalities, there were T18s (2 cases), tiploidy (1 case), 47, XXY (1 case), and T21 (1 case). Moreover, in one case, we identified a marker chromosome, and, in another, we found an increase in the centromeric heterochromatin of chromosomes 15, which was considered a normal variant. Mosaic trisomy 21 was determined by FISH in the T21 case, while the conventional cytogenetic method revealed an extra chromosome 21 in all metaphases.

Table. Indications and results of the patients.

Case No.	Age	Indication	Pregnancy week	Cytogenetic results	FISH results
1	28	TST/ DS	20	46,XX	N,XX
2	30	TST /DS	21	46,XX	N,XX
3	33	TST /DS	20	46,XY	N.XY
4	26	TST /DS	21, 4/7	46,XX	N,XX
5	33	TST /DS	19, 6/7	46,XY	N,XY
6	33	TST /DS	18, 4/7	47,XXY	N,XXY
7	30	TST /DS	18	46,XX	N.XX
8	29	TST /DS	17	46,XX,15cenh+pat	N,XX
9	34	TST /DS	17, 2/7	46,XX	N,XX
10	22	TST /DS	18, 1/7	46,XX	N,XX
11	34	TST /DS	18, 5/7	46,XX	N,XX
12	19	TST /DS	22	46,XX	N,XX
13	33	TST /DS	16, 2/7	46,XX	N,XX
14	27	TST /DS	21, 5/7	46,XY	N,XY
15	34	TST /DS	21	46,XX	N,XX
16	30	TST /T 18	18, 1/7	46,XY	N,XY
17	34	DST /T 18	17, 3/7	46,XY	N,XY
18	24	DST /T 18	19, 1/7	69,XXY	Triploidy, XXY
19	35	TST/DS+AMA	16, 1/7	46,XX	N,XX
20	35	TST/DS+AMA	16, 1/7	46,XX	N,XX
21	37	TST/DS+AMA	17	46,XX	N,XX
22	39	TST/DS+AMA	19, 2/7	46,XY	N,XY
23	38	AMA	21, 5/7	46,XY	N,XY
24	37	AMA	23	46,XX	N,XX
25	37	AMA	22, 5/7	46,XY	N,XY
26	36	AMA	19	46,XY	N,XY
27	41	AMA	17, 3/7	46,XY	N,XY
28	35	AMA	18, 1/7	Culture Failure	N,XY
29	37	AMA	20, 4/7	46,XX	N,XX
30	37	AMA	19, 1/7	Culture Failure	N,XX
31	37	AMA	16, 5/7	46,XX	N,XX
32	41	DS History of prior child	22, 4/7	46,XX	N,XX
33	31	DS History of prior child	16, 6/7	46,XY	N,XY

Table (continued).

Case No.	Age	Indication	Pregnancy week	Cytogenetic results	FISH results
34	32	IUGR, posterouretral valve	18	46,XY	N,XY
35	26	Oligohydramnios	19	46,XX	N,XX
36	24	IUGR	26	47,XY,+mar/46,XY	N,XY
37	27	Symmetric IUGR	26, 1/7	46,XY	N,XY
38	25	Mass in placenta Cardiac anomaly	29, 6/7	46,XX	N,XX
39	27	Short femur, polyhydramnios	19	46,XX	N,XX
40	34	Increased nuchal translucency, short femur	23, 2/7	46,XY	N,XY
41	21	Cardiomegaly, pericardial effusion, nonimmune hydrops	22	46,XX	N,XX
42	26	CAM in fetal lungs	18 1/7	46,XY	N,XY
43	31	Umbilical cyst	16, 1/7	46,XY	N,XY
44	30	Umbilical cyst	24	46,XY	N,XY
45	29	IUGR	16	46,XX	N,XX
46	30	Mild ventriculomegaly	26, 4/7	46,XY	N,XY
47	27	Early IUGR, mild ventriculomegaly	21, 1/7	46,XY	N,XY
48	24	IUGR, Choroid plexus cyst, renal agenesis	24	47,XX,+18	Trisomy 18,XX
49	35	Vermian agenesis	19, 5/7	46, XY	N,XY
50	25	Micrognathia, VSD, choroid plexus cyst, rocker bottom feet	21, 5/7	47,XY,+18	Trisomy 18, XY
51	27	Renal pyelectasy, complete atrioventricular septal defect	24	47,XX,+21	Mosaic trisomy 21 (%66)

N: Two signals for chromosome 13, 18, 21 each; AMA: Advanced Maternal Age; TST: Triple Screening Test; DST: Dual Screening Test; DS: Down Syndrome; IUGR: Intra-Uterine Growth Retardation; CAM: Cystic Adenoid Malformation; VSD: Ventricular Septal Defect

Discussion

Chromosome abnormalities mostly occur together with congenital abnormalities. They affect intelligence and usually there is no possible treatment. Given these facts, prenatal diagnosis of chromosome abnormalities is important. Despite the fact that standard conventional cytogenetic methods are indispensable, new techniques that assist diagnosis, especially those regarding aneuploidy screening, are used in routine practice today. Aneuploidy screening by FISH has also become a routine method in centers that perform prenatal diagnosis (4).

Standard cytogenetic analysis performed on fetal cell samples identifies chromosome aneuploidies and rearrangements with approximately 99.5% accuracy. Yet, the dependence of the method on live and reproducible cells could be a restraining factor on chromosome extraction (5). Fetal cells may not adapt themselves to the environment with a probability of 2%, and they do not enter mitosis. As a result of long-term cultures, chromosomes can be extracted and analyzed in 7 days at the minimum, and mostly on the 21st day. Especially in the advanced weeks of pregnancy, families with abnormal fetuses lose the option to terminate the pregnancy as the fetus reaches its viability limit.

Studies show that aneuploidies related to chromosomes 13, 18, 21, X and Y amount to 69%-80% of all chromosome abnormalities that are identified in the amniotic fluid (5). Pergament et al. argued that although chromosome abnormality incidence is 3.4% in cases of advanced maternal age, when no aneuploidies were detected for chromosomes 13, 18, 21, X and Y, the incidence decreases to 0.5% (6). They also showed that a similar decrease is observed in cases where abnormalities are identified in fetal USG and DS risk in screening tests.

The assessment of chromosomes 13, 18, 21, X and Y for aneuploidy by FISH could be completed in 2 days following the invasive intervention. This period is

considerably short when compared to the standard method. Such an advantage arises from the fact that interphase nuclei can be examined without the need to extract the chromosomes. In this respect, the method is also suggested to families that do not accept a secondary invasive procedure in cases where culture failure occurs. In such a case, however, the family should be informed about the advantages and disadvantages of the method in a comprehensible way in view of the risky situation. In our study, cultures that were intended for prenatal genetic diagnosis due to AMA were not successful and the chromosomes could not be extracted in 2 cases. The situation was explained in a second round of genetic counseling to both families, and the families that did not accept a second invasive intervention were informed about aneuploidy screening by FISH. Aneuploidy of any of the 5 chromosomes was not detected in the fetuses whose fetal USGs were within the normal limits, and the pregnancies resulted in healthy births.

DST is used for assessing neural tube defects, DS and T18, in the first 3 months of pregnancy (7). Thereby, it is planned to assess the risk in earlier weeks of pregnancy and to achieve the opportunity for early intervention in cases of abnormality. However, since most pregnancies that start with aneuploidy result in spontaneous miscarriage in the first 3 months of pregnancy, this attempt for early intervention may become unnecessary. In one of our patients, in whom we performed aneuploidy screening upon the risk of T18 in DST, we detected triploidy with both methods. It should be noted that the increase in T18 risk goes along with triploid pregnancies (8).

TST provides the opportunity for evaluating trisomy 21, trisomy 18 and neural tube defects by means of the serum samples taken from the mother in the 14^{th}-21^{st} weeks of pregnancy (9,10). Results for trisomy 21 and 18 are evaluated with 60% accuracy and 5% and 0.4% false positivity, respectively (10,11). Some 30%-40% of the fetuses with DS could not be identified in the first and second trimesters although routine screening tests were performed. It is noted that fetuses with DS can be identified at an accuracy rate of 65%-75% and false positivity rate of 4%-15% by prenatal ultrasonographic assessments in the second trimester (11). It is obvious that the combined use of maternal age, the screening test results and ultrasonographic assessment would yield more accurate results since it would allow a patient-specific approach.

In our cases, no DS or T18 was identified in any of the pregnancies in which DS and T18 risks were detected in TST. In one case with DS risk, 47, XXY karyotype was detected (case number 6). It is noted that, in Klinefelter Syndrome, DS-like results can be obtained by TST (9). In TST, an increase in beta-HCG level is observed in the presence of a significant increase in the level of AFP and vacuolized trophoblasts in oligohydroamniosis (9). Therefore, detection of the DS risk in TST requires attention with regard to cases other than aneuploidies that put the pregnancy at risk.

Although FISH provides results with almost 100% accuracy for aneuploidy screening, the same method is insufficient in detecting 25%-30% of cytogenetic abnormalities that can be identified in routine practice (12). Only numerical changes in the 5 chromosomes examined can be determined with this method. Rearrangements of these chromosomes and numerical and structural changes in other chromosomes are excluded from the assessment. Furthermore, decreased success of direct FISH due to the reduction of the cell quality in the advanced weeks of pregnancy and especially due to contamination of the amniotic fluid with maternal blood is a significant problem faced in practice (13).

In our study, in 1 of the 2 cases in which no aneuploidy was detected by FISH, mosaic marker chromosome was identified by the conventional cytogenetic method, while in the other an increase in the centromeric heterochromatin of the 15^{th} chromosome, which is considered a normal variant, was identified by the same method (case number 8 and 36, respectively). In the first case, it was shown that the variant chromosome was transferred from the father. The marker chromosome detected in the second case was identified as mosaic in similar rates in CS and in the peripheral blood sample after birth. This pregnancy resulted in premature birth in the 28^{th} week, and the baby was lost in the 28^{th} day due to necrotizing enterocolitis, right atrial thrombus, and sepsis.

In cases in which aneuploidy risk is identified by AMA and screening tests that are applied to the maternal serum, early diagnosis can be achieved by means of aneuploidy screening performed with FISH and thereby families can be assisted in making their decisions as to whether to continue the pregnancy. However, this method is inadequate for pregnancies that are at risk due to other chromosomal diseases in addition to aneuploidy.

In terms of aneuploidy screening, our research yielded conformity of 98.03% between the results of the 2 methods. Mosaic trisomy 21 that we detected in case number 51 by FISH was found to be pure by the conventional cytogenetic method subsequently. Moreover, the conventional cytogenetic research conducted on the skin sample taken after the pregnancy was terminated upon the decision of the family did not verify mosaicism. We assumed that the mosaicism detected with FISH might be either because of the contamination of the amniotic fluid with maternal blood, or due to the inadequacy of hybridization resulting from the failure of the probe to reach the cells with insufficient quality. Detection of the signals of both the X and Y chromosomes in all cells examined after hybridization with 18, X and Y probes indicates that the situation was due to hybridization inadequacy specific to the probe rather than maternal contamination.

Consequently, aneuploidy screening of uncultured amniotic cells with direct FISH is important for prenatal diagnosis in pregnancies that have a high risk of aneuploidy. In cases in which suspicious results are obtained, examination of the fetus with detailed USG is a supplementary approach. Since FISH has its own limitations, it would be more appropriate to use it as a supplementary method in routine practice along with conventional cytogenetic methods, rather than viewing it as a method that can replace the latter.

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